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Mercury Methylation from Unexpected Sources: Molybdate-Inhibited Freshwater Sediments and an Iron-Reducing Bacterium

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Methylmercury has been thought to be produced predominantly by sulfate-reducing bacteria in anoxic sediments. Here we show that in circumneutral pH sediments (Clear Lake, CA) application of a specific inhibitor of sulfate-reducing bacteria at appropriate concentrations typically inhibited less than one-half of all anaerobic methylation of added divalent mercury. This suggests that one or more additional groups of microbes are active methylators in these sediments impacted by a nearby abandoned mercury mine. From Clear Lake sediments, we isolated the iron-reducing bacterium *Geobacter* sp. strain CLFeRB, which can methylate mercury at a rate comparable to *Desulfobulbus propionicus* strain 1pr3, a sulfate-reducing bacterium known to be an active methylator. This is the first time that an iron-reducing bacterium has been shown to methylate mercury at environmentally significant rates. We suggest that mercury methylation by iron-reducing bacteria represents a previously unidentified and potentially significant source of this environmental toxin in iron-rich freshwater sediments.

Mercury has become a global concern due to its toxic properties and has contaminated water sources through atmospheric deposition, weathering of cinnabar, runoff from industrial sites and abandoned mines, and microbial production of acid-rock drainage. In California, acid-rock drainage from mercury deposits in the Coast Range and mercury used in the Sierra Nevada foothills for gold recovery are the dominant sources of water contamination. Clear Lake, a eutrophic lake in the mercury belt of the California Coast Range, receives acid-rock drainage from the Sulfur Bank Mercury mine located on the northeastern edge of the lake. There sediment concentrations of mercury can exceed 400 ppm and decline exponentially with distance from the mine (58, 59). In Clear Lake food chain bioaccumulation caused mercury to reach levels in fish tissues (>95% as methylmercury) that triggered a state health advisory limiting consumption of 10 fish species (16).

Mercury is converted to methylmercury in anoxic sediments (72) via incompletely characterized mechanisms that are classically attributed to sulfate-reducing bacteria (12, 17). This conclusion principally rests on the observation that estuarine sediments known to methylate exogenous mercury failed to do so when incubated under oxic conditions or in the presence of molybdate, an inhibitor that disrupts the central energy metabolism of sulfate-reducing bacteria. Sulfate-reducing bacteria have been regarded as the principal methylators in both marine and freshwater sediments, with no contribution consistently ascribed to other metabolically defined groups of *Bacteria* or *Archaea*. Recently, in certain riverine sediments from the southeastern United States a substantial portion of biological potential for mercury methylation could be attributed to activity of organisms other than sulfate-reducing bacteria (67). Because these sediments contained iron minerals and because reduction of iron was the dominant terminal electron accepting process, methylation was postulated to be due to the activity of iron-reducing bacteria. Earlier pure-culture studies offered limited support for this hypothesis. Although three strains of iron-reducing bacteria were reported to methylate mercury (C. C. Gilmour, G. S. Riedel, J. D. Coates, and D. Lovley, Abstr. 96th Gen. Meet. Am. Soc. Microbiol., abstr. O-15, 1996), they did so at apparent rates less than a few percent of those reported for active sulfate reducers.

Here we show that sediments from Clear Lake, CA, which contain dissolved iron and show signatures of iron reduction, continue to produce methylmercury even in the presence of molybdate concentrations sufficient to fully inhibit sulfate reduction. Importantly, the first iron-reducing bacterium we isolated from these sediments, *Geobacter* sp. strain CLFeRB, is able to methylate mercury in pure cultures at rates comparable to those of sulfate-reducing bacteria. Finally, properties of iron-reducing bacteria that increase their possible impact on methylation in freshwater sediments are also discussed.

MATERIALS AND METHODS

Sampling site and sediment collection. All sediments were collected from Clear Lake, CA, a mercury-contaminated, moderately eutrophic lake in the Coast Range of northern California. Samples were collected from four sites, UA-03 (39°3.65′N, 122°51.02′W), OA-04 (39°0.69′N, 122°42.01′W), OA-15 (39°0.26′N, 122°40.29′W), and OA-15+7W (located approximately 7 m west of site OA-15), either by Eckman dredge (surface area, 0.056 m^2) or by hand coring by a diver. All replicates came from subcores from the same Eckman grab or were taken side by side by a diver. Samples were transported vertically to the laboratory on wet ice, stored at 4°C, and manipulated within 4 days.

Effect of molybdate on sulfate reduction rates and mercury methylation by native microbial communities. Lake sediments were collected at sites OA-04 and UA-03 from a boat using an Eckman dredge, which penetrated to a depth of roughly 15 cm. Concurrent with each collection, water temperature 0.5 m above the sediment-water interface was recorded. On the boat, sediments were subsampled to a depth of at least 6 cm using Teflon core tubes with a minimum of 2 cm of overlying water also retained. Cores were capped (black butyl rubber stoppers) and transported as described above. Experiments were initiated by mixing 1 part of sediment (upper 6 cm, unless noted) with 2 parts of lake water

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that had been filtered and sparged with N_2 gas that had been passed over hot copper shavings to remove traces of oxygen. After each "master slurry" was supplemented with $HgCl_2$ (typically 85 ppm Hg^{2+}, double that for August of year 2), replicates (5 or 10 ml) were transferred to separate glass jars, supplemented with [^{35}S]sulfate and/or molybdate as needed, closed with butyl rubber stoppers, and incubated at in situ temperature. Throughout the entire process anoxic conditions were preserved using a modified Hungate technique (69). When replicates were tested, each came from a slurry derived from a separate core. Typically, duplicate "time-zero" slurries were frozen immediately after supplementation with divalent mercury, and methylmercury concentrations determined for these samples were used to correct rates determined from duplicate end points of each incubation.

In October of year 1, the effect of 0.2 mM molybdate (versus control) was explored for mercury methylation in slurries from three depths (0 to 2, 2 to 4, and 4 to 6 cm) at each site. Incubations were for 120 h at 16°C. When no significant trend versus depth was observed, the single data points corresponding to each site-depth combination were averaged ($n = 3$) to yield a rate determination for each site. In years 2 and 3 the tabulated impact of molybdate was determined as follows: April, 0.20 mM molybdate and 4- and 60-h incubations at 13°C; June, 0.10 and 0.25 mM molybdate and 67-h incubations at 17°C; August, 0.20 mM molybdate and 60 h incubations at 23°C; October, 0.10 and 0.40 mM molybdate and 70-h incubations at 19°C. When necessary, linear interpolation was used to estimate the tabulated rate corresponding to 0.20 mM molybdate.

ICP-MS and elemental analysis. Sediment cores taken from sites OA-04, OA-15, and OA-15+7W were transported and manipulated at 4°C. The upper 6 cm of each core was transferred to a separate acid-washed Teflon bottle and centrifuged at 4°C for 10 min at 3,000 × *g*. Supernatant pore water was filtered through Whatman glass microfiber filters (GF/F), and a portion of the water (1.5 ml) was used for analysis of pH. To the remainder of each pore water sample, 6 M HNO_3 (trace metal grade; Sigma) was added at a concentration of 13.4 μl per 1 ml of pore water, and the samples were stored at 4°C in butyl rubber crimp-sealed, acid-washed bottles until analysis. After 20-fold dilution with the same strength nitric acid (trace metal grade; Fisher) samples were analyzed for metals by inductively coupled plasma mass spectrometry (ICP-MS) under robust plasma conditions (Agilent 7500i; very low molecular oxides as determined by a CeO/Ce ratio of <0.4%) with a Babbington nebulizer serving a thermoelectrically cooled spray chamber.

Sulfate concentrations were determined by suppressed ion chromatography using a conductivity detector, OmniPac PAX-500 guard and analytical columns, and an anion trap column (Dionex).

Media. All media, medium components, and cultures were manipulated using a modified Hungate technique as described by Widdel and Bak (69). The headspaces of all anaerobic tubes and bottles were flushed with an oxygen-free CO_2-N_2 gas mixture (10%/90%) and sealed with butyl rubber stoppers. All media were used directly or stored sterile in the dark at room temperature (for a maximum of 3 months) until use. Cells were grown with shaking 15 min every 12 h at 28°C unless otherwise specified.

Growth media. Two types of media were used to isolate and propagate iron-reducing bacteria: a ferrihydrite medium (with solid ferric oxyhydroxide as the electron acceptor) and a ferric citrate medium (with soluble ferric citrate as the electron acceptor). Both media contained the following (per liter): salts (0.25 g NH_4Cl, 0.2 g KH_2PO_4, 0.4 g $MgCl_2 \cdot 6H_2O$, 0.15 g $CaCl_2 \cdot 2H_2O$, 1.0 g NaCl, and 0.5 g KCl), sodium bicarbonate (30 mM, final concentration), sodium cysteine (2.4 mM, final concentration), sodium acetate (10 mM, final concentration), and sulfate-free nonchelated trace element mixture (1 ml of the formulation of Widdel and Bak [69]). Vitamins (filter sterilized) were added to the following concentrations (per liter): 4-aminobenzoic acid, 0.4 μg; D-(+)-biotin, 0.1 μg; nicotinic acid, 1 μg; sodium D-(+)-pantothenate, 0.5 μg; pyridoxine dihydrochloride, 1.5 μg; thiamine, 1 μg; vitamin B_{12}, 0.5 μg (69); riboflavin, 5 μg; folic acid, 0.3 μg; lipoic acid, 0.1 μg. The final (postautoclaving) pH of each medium was adjusted to 7.2 using either 1 M HCl or 1 M NaOH. For the ferrihydrite medium (used for initial enrichments, preliminary methylation experiments, growth curves, and determination of methylation rates), iron oxyhydroxides were synthesized as described elsewhere (13a) and added at 30 mM. Ferric citrate medium (used for culture purification, growth for DNA isolation, and preliminary methylation experiments) contained ferric citrate at 33 mM and was neutralized by the addition of 30 ml of 1 M Na_2CO_3. Anaerobic shake tubes, used to obtain the pure culture, were made with 3 ml 1.1% Difco Bacto agar and 6 ml of ferric citrate media in Hungate tubes. *Desulfobulbus propionicus* strain 1pr3 was grown on DSMZ medium 194 (http://www.dsmz.de/media/med194.htm) with the following modifications: sodium propionate was replaced with of sodium lactate (10 mM final concentration), and trace elements (1 ml per liter) were those of Widdel and Bak (69).

Isolations and strains. The inoculum for isolation of an iron-reducing bacterium was obtained from site OA-15+7W, which is a moderately mine-impacted sediment (pH 6.15). The core was collected at a water depth of approximately 5 m at a site 30 m offshore of the graded waste rock piles adjacent to the Sulfur Bank Mercury mine. Sediment was stored in a glass container under a nitrogen atmosphere at 4°C before processing. An enrichment was made with 1 ml of sediment from the top 6 cm and 50 ml of anaerobic ferrihydrite medium. Growth was judged by a blackening of the medium and production of ferrous iron, which was roughly quantified by addition of 0.1 ml of medium to 0.9 ml of 1 M HCl and then spotted onto test strips (EM Quant Iron [Fe^{2+}] test; Merck KGaA). After several sequential transfers, the positive enrichment was diluted in anaerobic agar shake tubes of ferric citrate medium. Colonies and cells were examined (dissecting scope and phase-contrast, respectively) and transferred until both were judged to be uniform.

D. propionicus strain 1pr3 was obtained from American Type Culture Collection (ATCC 33891) and was grown in modified DSMZ medium 194 as described above.

Sequences and trees. DNA for sequencing the 16S rRNA gene of strain CLFeRB was obtained from cells grown to stationary phase in 15 ml of ferric citrate medium. The cells were pelleted by centrifugation at 4°C for 10 min at 7,700 × *g*, washed once with 10 ml saline, and recentrifuged. Cells were lysed by boiling for 5 min in 0.2 ml lysis buffer (Tris, pH 8, 10 mM EDTA, Triton X-100). The lysate was extracted twice with equal volumes of chloroform. The aqueous phase (10 μl) was used in PCR amplification with Ampli*Taq* Gold (Applied Biosystems), using primers 8fpl and 1492rpl (68) under the following conditions: initial denaturation at 95°C for 4 min; 35 cycles of 95°C for 1 min, 45°C for 2 min, and 72°C for 2 min; and a final extension at 72°C for 20 min. The product was cleaned using the QIAquick PCR purification kit (QIAGEN) per the manufacturer's protocol. The purified product was sequenced by the University of California at Davis's DBS sequencing facility on an ABI sequencer. The sequence was assembled using Clone Manager (Scientific and Educational Software) and compared to sequences of high similarity in GenBank using Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nih.gov/BLAST/) to determine strain CLFeRB's potential affiliation. The resulting strain CLFeRB sequence was imported into the ARB program (40) (http://www2.mikro.biologie.tu-muenchen.de/arb/) and aligned to the ssu_jan03.arb 1,000- and 1,400-base pair database (http://www2.mikro.biologie.tu-muenchen.de/download/ARB/data/) using the automatic alignment tool followed by manual alignment. Phylogenetic trees and bootstrap values were determined with aligned sequences using the distance matrix with minimum evolution and maximum likelihood of trees in PAUP* 4.0 (61).

Growth curves and methylmercury analysis. To examine the growth and methylation rates for strain CLFeRB, a 1% inoculum was added to 250 ml of anoxic ferrihydrite medium. Sulfate-reducing bacterium *D. propionicus* strain 1pr3 was added as a 1% inoculum to 500 ml of modified DSMZ medium 194. Both cultures were incubated under strictly anoxic conditions in butyl rubber-stoppered flasks (twice the medium volume) at 28°C. Each contained a borosilicate glass stir bar, which was used for magnetic stirring for 15 min every 12 h. Mercuric chloride was added to inoculated media at a final concentration of 1 ppm, and this concentration was confirmed by atomic absorption spectroscopy (DMA-80 direct mercury analyzer; Milestone Srl, Sorisole, Italy). Controls included autoclaved stationary-phase cultures with 1 ppm mercuric chloride added postautoclaving and stationary-phase cultures lacking added mercury. Samples for methylmercury analysis (25 to 75 ml) were transferred to acid-washed Teflon bottles, acidified to a 0.5% final concentration with concentrated hydrochloric acid (trace metal grade; Sigma), and stored at −20°C until analysis. Methylmercury was distilled (25), ethylated to form a volatile methyl ethylmercury derivative, and flushed into a graphite carbon trap to preconcentrate the analyte and remove interfering compounds. Samples were isothermally chromatographed, pyrolytically converted to elemental mercury, and analyzed by cold vapor fluorescence detection. Results were corrected for distillation efficiency. These determinations were made by Batelle Marine Sciences Laboratory in Sequim, Washington, using U.S. EPA draft method 1630 (6, 21). Direct cell counts for strain CLFeRB in ferrihydrite medium were achieved by preserving cells in glutaraldehyde (1.5%, final concentration), dissolving particulate iron in a sodium dithionite solution as described by Thamdrup et al. (64), and filtering onto an Irgalan Black-B prestained 0.2-μm Nuclepore filter (64). Immediately postfiltration, cells were stained (2 min) with 0.01% acridine orange and counted. *D. propionicus* strain 1pr3 cells were sampled, diluted in filter-sterile saline, immediately stained with 0.01% (final concentration) acridine orange, and filtered onto an Irgalan Black-B-prestained 0.2-μm Nuclepore filter. All cells were counted with epifluorescence microscopy (23). The specific growth rate (26) was calculated using a least-squares method on log-transformed data. The following

TABLE 1. Inhibition[a] by MoO_4^{2-} (0.20 mM) of the rates of sulfate reduction and mercury methylation in anaerobic slurries of sediments from Clear Lake, CA

Expt date (mo, yr)	Site OA-04			Site UA-03		
	Avg ambient concn of SO_4^{2-} (mM)	Inhibition (%) of:		Avg ambient concn of SO_4^{2-}	Inhibition (%) of:	
		SO_4^{2-} reduction	Hg methylation		SO_4^{2-} reduction	Hg methylation
October, 3	0.08	82	14	0.04	ND[b]	ND
August, 2	0.05	96	36	0.05	77	15
June, 2	0.09	94	ND	0.08	90	ND
April, 2	0.09	92	ND	0.08	76	ND
October, 1	0.08	ND	46	0.06	ND	69

[a] Inhibition measured as percent reduction of rate in the presence of molybdate versus the rate for a control incubation lacking molybdate.
[b] ND, not done.

equation (29) was used to calculate the methylation rate: $K_{meth} = [CH_3Hg^+]_t k/(x_t - x_0)$, expressed as attomol of methylmercury cell^{-1} day^{-1}, where $[CH_3Hg^+]_t$ is the net methylmercury produced over the period of exponential growth, k is the calculated specific growth rate, and x_0 and x_t are, respectively, initial and final cell numbers.

Nucleotide sequence accession number. The 16S rDNA gene sequence for *Geobacter* sp. strain CLFeRB has been deposited in GenBank under accession number DQ086800.

RESULTS

Effects of molybdate on mercury methylation and sulfate reduction. For site OA-04 addition of molybdate at a concentration of 0.20 mM, which was roughly equal to that of ambient sulfate, inhibited sulfate reduction by 82 to 96% in four separate experiments (Table 1). The corresponding inhibition of mercury methylation was less, only 14 to 46% over three experiments. For site UA-03 this same concentration of molybdate inhibited sulfate reduction by 76 to 95% (three experiments; Table 1) but inhibited mercury methylation by only 15 to 69%. There was no obvious trend in percent inhibition as a function of season, and the decoupling between inhibition of sulfate reduction and mercury methylation was observed over a range of molybdate concentrations beyond 0.2 mM. For the first two experiments of Table 1 (October, year 3; August, year 2) replicates were tested at concentrations ranging from 0.05 to 0.40 mM and even the lowest molybdate concentrations (0.05 to 0.10 mM) resulted in at least 68% inhibition of sulfate reduction while mercury methylation was inhibited by only 0% to 29%. At the highest molybdate concentration tested (0.40 mM), sulfate reduction was inhibited by at least 90% ($n = 3$) while mercury methylation was inhibited by only 28 to 53%.

Elemental analysis of sediment pore water. Sediments collected from Clear Lake were of three types: "white floc" sediment (site OA-15) with an acidic pH (~4), characterized by an obvious white flocculent material that forms when acid-rock drainage contacts slightly alkaline lake water (59); "black floc" sediment (site OA-15+7W), with a slightly acidic to neutral pH (5 to 7), containing black flocculent material that reflects active sulfate reduction in the presence of iron and other metals; and normal lake sediment (site OA-04), which has a slightly alkaline pH (7 to 8), with no visible flocculent material or blackening. The precise coordinates and extent of sediment characterized by white or black floc varied with season (59), but the sediment was generally localized within 25 to 35 m of the lake shore adjacent to the Sulfur Bank Mercury mine. Based on pH, soluble sulfate, and filterable aluminum concentrations (Table 2), the impact of acid-rock drainage is greatest at site OA-15, next strongest at site OA-15+7W, and least at site OA-04, which is located roughly 0.5 km from the mine site. Sulfate reduction appears to be suppressed by the low pH of site OA-15 (D. C. Nelson, unpublished observations); hence all divalent cations are elevated there relative to the other sites. By contrast, 7 m away (site OA15+7W) sulfate reduction is most active and the resultant sulfide has, via formation of metal sulfide precipitates, decreased the concentration of divalent metal ions (Fe, Mg, Co, Ni, and Zn) to a level below or equal to those of control site OA-04. Nonetheless, aqueous Fe and Mn are clearly present in the system, indicating reduction of solid-phase Fe(III) and Mn(IV).

Isolation and phylogenetic analysis. Inoculation of 1 g of mine-impacted sediments (from site OA-15+7W, pH 6.15) in ferrihydrite medium for 1 month at 25°C produced roughly 100 mg/liter of ferrous iron. From this enrichment repeated dilution and purification in ferric citrate agar deeps yielded a pure culture characterized by pink lenticulate colonies that altered the adjacent medium from green to colorless. Strain CLFeRB is characterized by cells that are vibrioid motile anaerobes.

TABLE 2. Elemental analysis of sediment pore water from Clear Lake, CA

Site	pH	Concn[a] (ppm) of:									
		Mn	Fe	SO_4^{2-}	Al	Mg	Zn	Hg	Co	Ni	Cr
OA-15	4.1	17.44	63.69	2,547–13,248	184.31	172.31	1.12	0.00035	0.62	1.09	0.015
OA-15+7W	5.4–7.4	0.66	1.23	78–2,154	1.47	19.76	0.30	0.00043	0.001	0.002	0.002
OA-04	7.7	3.84	1.04	2–4[b]	0.05	71.61	0.92	0.000035	0.001	0.003	0.006

[a] Elemental concentrations for all except SO_4^{2-} were determined by ICP-MS, with values reported representing the differences between samples and water blanks. Concentrations of SO_4^{2-} were measured by ion chromatography.
[b] SO_4^{2-} range reported is from several nearby sediment sites.

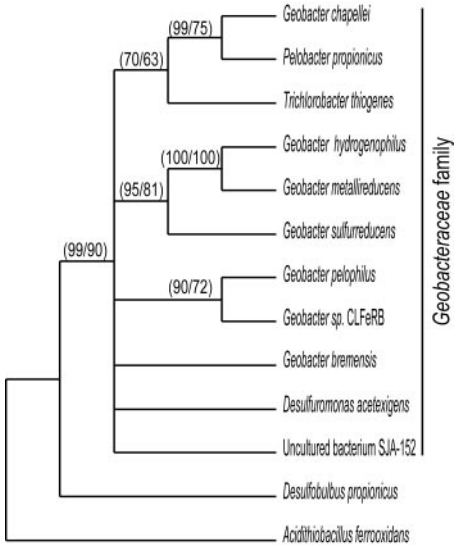

FIG. 1. Distance matrix cladogram based on an alignment of the 16S rRNA gene of dissimilatory iron-reducing bacteria including *Geobacter* sp. strain CLFeRB and select *Proteobacteria*. *Acidithiobacillus ferrooxidans*, a bacterium from the γ-subclass of the *Proteobacteria*, was included as an outgroup. For bootstrap analysis starting trees were created using stepwise addition with random addition sequence and the HKY85 substitution model. Paired numbers on nodes (percentages) represent bootstrap values greater than 50% for a distance matrix with minimum evolution and maximum-likelihood analyses and were generated using fast-heuristic search (1,000 and 100 replicates, respectively).

Growth was not inhibited by 1 mM sodium molybdate either in the presence or absence of 1 mM sodium sulfate.

Phylogenetic analyses of 1,397 homologous base pairs of the 16S rRNA gene sequence placed strain CLFeRB in the genus *Geobacter* within the δ-subclass of the *Proteobacteria*. A distance likelihood tree inferred with the HKY85 substitution matrix is shown (Fig. 1), and a maximum-likelihood analysis inferred using the same substitution matrix produced a similar tree. All cultured strains represented in this tree are reported to reduce ferric iron, but not all seem to have been tested for the ability to utilize insoluble ferric oxyhydroxides (38). The bootstrap values grouping strain CLFeRB with the family *Geobacteraceae* are 99% and 90% for distance and maximum-likelihood methods, respectively. The highest sequence similarity of strain CLFeRB is to *Geobacter pelophilus* (97%), and CLFeRB clustered with this species in 90% and 72% of generated tree replicates using methods described above.

Growth and methylmercury production rates. Initial experiments with strain CLFeRB showed that, in medium containing 1 ppm of $HgCl_2$, 16 days of growth produced 11.2 nM of methylmercury in particulate ferrihydrite medium or 5.9 nM of methylmercury in soluble ferric citrate medium at 25°C. It was not determined whether final cell densities differed for the two conditions. When grown in the absence of mercury at 28°C (ferrihydrite medium), this strain had a specific growth rate of 2.85 day^{-1}, but addition of 1 ppm mercuric chloride slowed growth to 1.45 day^{-1}. The least-squares method (see Materials and Methods) yielded a methylation rate of 0.076 attomol $cell^{-1}$ day^{-1} for strain CLFeRB (Fig. 2A). For comparison, the sulfate-reducing bacterium *D. propionicus* strain 1pr3, which is known from the literature to be an active methylator, was tested; without the addition of mercury at 28°C it had a specific growth rate of 1.90 day^{-1}. The presence of 1 ppm of mercuric chloride lowered the growth rate to 1.29 day^{-1}, and methylmercury was produced at 0.043 attomol $cell^{-1}$ day^{-1} (Fig. 2B). Killed and "no-added-mercury" controls showed no net production of methylmercury for either bacterium.

DISCUSSION

Based on a substantial number of molybdate inhibition studies by others, sulfate-reducing bacteria have been judged to be the dominant methylators of mercury in marine, estuarine, and freshwater sediments (9, 11, 12, 17, 18, 31, 33–35, 45, 50). Furthermore this conclusion is widely cited in related literature (2, 4, 5, 8, 10, 15, 19, 28, 29, 41, 43, 49, 51, 65). The present study raises questions about this paradigm in two different ways. First, we repeatedly investigated sulfate-impacted, iron-impacted, circumneutral pH sediments from two sites in a northern California lake for the effects of molybdate on sulfate reduction and mercury methylation. Our finding (Table 1) is that a molybdate concentration high enough to eliminate vir-

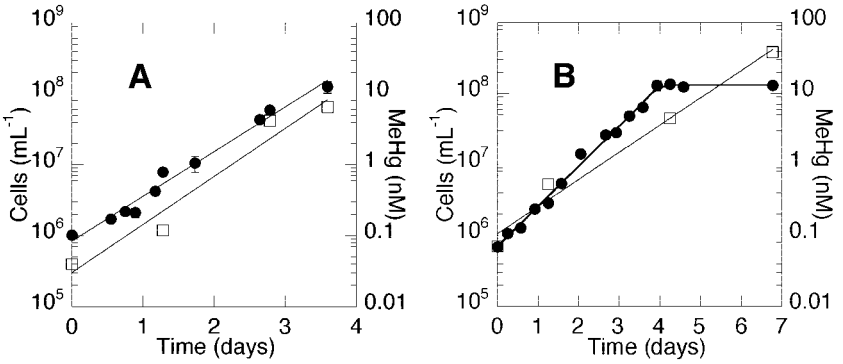

FIG. 2. Cell density (circles) and methylmercury (MeHg) concentration (squares) for (A) strain CLFeRB with time and for (B) *D. propionicus* strain 1pr3 with time. Error bars represent one standard deviation of the mean (standard error, $n = 3$) and were calculated for cell density at all time points. Methylmercury from uninoculated controls and autoclave-killed controls (not shown) was below the detection limit for both methylating organisms.

tually all sulfate reduction activity generally inhibited less than one-half of total sediment activity for mercury methylation. Secondly, the first iron-reducing bacterium we cultured axenically from nonacidic pH sediment of this lake methylated mercury more actively (per cell, per day) than a sulfate reducer that was tested in parallel and that was known from the literature to be an active methylator. What follows is an attempt to reconcile our findings with the historical and recent literature on methylation by natural bacterial populations in mercury-impacted marine and freshwater sediments.

Mercury methylation and molybdate inhibition in situ. Inhibition of dissimilatory sulfate reduction with molybdate in whole cells of *Desulfovibrio desulfuricans* was originally described by Peck (53, 54). In this role molybdate acts as a competitive inhibitor for sulfate in the "sulfate activation" step of dissimilatory sulfate reduction, which is catalyzed by the enzyme ATP sulfurylase. The resultant molecule of adenosine-5′-phosphomolybdate, the synthesis of which consumes an ATP molecule, is unstable, and repeated production and breakdown of this intermediate can lead to a targeted "energy uncoupling" of sulfate-reducing bacteria (70). Researchers have suggested that the appropriate concentration for molybdate application to sediments is equimolar to that of ambient sulfate (48). The initial demonstration by Compeau and Bartha used estuarine sediments and applied molybdate at 20 mM, a concentration comparable to marine sulfate levels (12). Thus, the conclusion that sulfate-reducing bacteria dominate mercury methylation appears to be true at least for certain marine sediments (12, 36). In a number of freshwater systems, typically characterized by a sulfate concentration less than 0.2 mM (17), molybdate was applied at this same elevated concentration (18, 19), which is 100-fold higher than that found to inhibit the great majority of sulfate reduction in Clear Lake sediments. We speculate that application of molybdate at inappropriately high concentrations may have a general negative effect on assimilatory sulfate reduction in many types of bacteria, a process that also requires sulfate activation by ATP sulfurylase but proceeds at a small fraction of the rate of dissimilatory reduction. Hence, it seems reasonable to speculate that excessive molybdate concentrations, as applied in a number of other studies of freshwater sediments, might cause a more general inhibition of many sediment microbial processes. Our findings of only partial inhibition of mercury methylation (Table 1) by appropriate concentrations of molybdate match those of Winfrey and Rudd, (71), whose study is one of the few previous studies of freshwater sediments to explore the impact of a range of molybdate concentrations on methylation.

Physiologically defined microbial groups as potential methylators in freshwater sediments. A spectrum of organisms have been shown to methylate mercury in pure culture, including *Neurospora crassa*, *Clostridium cochlearium*, *Pseudomonas fluorescens*, *Enterobacter aerogenes*, *Desulfovibrio desulfuricans*, and *Desulfobacter* sp. strain BG-8 (12, 22, 34, 37, 52, 66, 73). However, some of these organisms are not involved in dominant terminal-electron-accepting processes in anoxic sediments and consequently probably have a limited contribution to in situ production of methylmercury. Methanogenic extracts have been shown to methylate mercury in the laboratory (72), but studies in the presence/absence of specific methanogen inhibitors have repeatedly shown that this group does not contribute significantly to methylation in mixed natural-sediment populations (12, 18, 50, 51). It is well established that sulfate-reducing bacteria can both grow and methylate mercury while living via fermentation in the absence of sulfate (2, 51). However, methylation and growth under these conditions were inhibited by the only concentration of molybdate tested (2.0 mM) (51), which argues against this process contributing to molybdate-independent methylation in sediments.

A number of iron-reducing bacteria and manganese-reducing bacteria in freshwater and in marine systems have been shown to withstand concentrations of molybdate up to 20 mM and to continue to oxidize organic carbon (7, 47, 57). Strain CLFeRB can grow in the presence of an intermediate concentration (1 mM) of molybdate, and the ability of this strain to methylate mercury in pure culture suggests the potential of it and other iron-reducing bacteria to account for at least a portion of the observed "molybdate-independent" methylation.

Iron, iron reduction, and methylmercury-producing sediments. Certain elements, e.g., Al, Mn, and Fe, found in elevated concentrations in Clear Lake pore waters (Table 2) often occur in natural waters as colloids, and their presence in Clear Lake has been confirmed by others (59). Such colloids have been found to co-occur with mercury and are responsible for its transport throughout Clear Lake (32, 58). In Clear Lake, deposition and recycling of these particulates via bioturbation can make trace elements such as mercury available at sediment depths of 10 to 30 cm (60). Colloids such as ferric and manganese oxyhydroxides are precipitated and dissolved by redox changes, thereby controlling the dissolution and release of sorbed Zn, Cu, Cd, Pb, and Hg (20, 44). The presence of soluble ferrous iron in Clear Lake sediment pore water suggests that active iron reduction occurs in the sediments at sites OA-15+7W and OA-04, but aqueous iron at site OA-15 cannot be unequivocally attributed to iron reduction due the possibility of both soluble ferric and ferrous iron being present in acidic sediments. The occurrence of free soluble ferrous iron despite the presence of sulfide from sulfate reduction emphasizes the potentially high rates of iron reduction occurring in these sediments. Reduction of Fe(III) and Mn(IV) can be controlled by either abiotic chemical reactions or through metal-reducing bacteria. The iron-reducing bacterium *Shewanella putrefaciens* strain 200 has been shown, via the reduction of ferric oxyhydroxides, to increase aqueous concentrations of previously sorbed Zn^{2+}, but this reaction is sensitive to the specific mineral composition (13). More broadly, the addition of ferric oxyhydroxides has been shown to increase methylation rates in certain lacustrine sediments (27). Warner et al. (67) demonstrated that freshwater sediments with iron reduction as the dominant process had methylation potentials similar to those of sediments in which sulfate reduction was the dominant terminal electron accepting process. Recent studies (46) showing stimulation of methylation by low concentrations of iron (0.3 and 3.0 mM) suggest (assuming some oxidation of the added ferrous chloride) that methylation by iron-reducing bacteria is also important in marine sediments. These increased methylation rates may be due to increases in the bioavailability of mercury for methylators in general, via dissolution of iron colloids or via electron acceptor stimulation of methylation by metal-reducing bacteria.

TABLE 3. Comparison of measured rates with methylation rates reported in the literature

Organism	Methylation rate(s)	Hg^{2+} added (ppm)	Reference or source
Strain CLFeRB	0.076[a]	1	This study
D. propionicus strain 1pr3	0.043,[a] 0.102[a,c]	1	This study
Desulfovibrio desulfuricans strain ND 132	0.027 ± 0.008[a], 0.020 ± 0.003[a]	NA[d] (cinnabar)	29
D. propionicus strain 1pr3	0.0049[b], 0.0037[b], 0.00047[b,c]	0.0008	2
D. propionicus strain 1pr3	0.00048 ± 0.0036[b,c]	0.1	34
Desulfovibrio desulfuricans subsp. *desulfuricans*	0.0047 ± 0.014[b,c]	0.1	34
Desulfobacterium sp. strain BG-33	0.69 ± 1.2[b,c]	0.1	34

[a] These values are reported as attomol methylmercury (MeHg) $cell^{-1}$ day^{-1} and were determined by the formula in Materials and Methods.
[b] These values were originally reported differently and for comparison were converted to attomol MeHg $cell^{-1}$ day^{-1}, and cell growth was calculated as a change in cell number rather than specific growth rate.
[c] These rates were determined during growth in late exponential/stationary phase.
[d] NA, not applicable.

Growth rates and methylation rates. When evaluated on a per-cell basis, strain CLFeRB methylated mercury more actively then *D. propionicus* strain 1pr3 (Table 3). These rates translate to one bacterial cell methylating 20 to 30 molecules of mercury per minute. For comparison, in a central physiological process, such as dissimilatory sulfate reduction, a cell reduces approximately 7×10^7 molecules of sulfate per minute during exponential growth. This reiterates a point made previously by others (15, 17), namely, that mercury methylation, whether in sulfate- or iron-reducing bacteria, is not likely to be associated with a central metabolic process. The methylation rates we measured for strain CLFeRB and for *D. propionicus* strain 1pr3 are of the same magnitude, but comparison of per-cell rates from the literature as a whole shows a much greater variation between organisms and between different researchers investigating a single strain such as *D. propionicus* strain 1pr3. For this strain there is a general trend for higher added mercury to correlate with higher per-cell methylation rates. Methylation among sulfate-reducing bacteria has been shown to vary between genera, and researchers have suggested that those able to utilize acetate ("complete oxidizers") methylate at higher rates (34). Although similar metabolic diversity exists among iron reducers (38), the range of rates and breadth of distribution of the capacity for methylation among these bacteria remain unexplored.

The growth phase of the culture analyzed sometimes adds to observed variation in methylation rates among sulfate-reducing bacteria. During exponential phase methylmercury increased proportionally with cell number for all strains tested (Table 3), but during stationary phase net methylmercury production decreased (2, 8) or increased (34). These differences may reflect differences in bioavailability of the divalent mercury due to differences in concentration and/or potential complexation with components in the growth medium. Added divalent mercury will not remain exclusively in the aqueous phase and may be sorbed onto mineral surfaces or precipitate with medium components or waste products such as sulfide (3, 28, 29). Various concentrations of mercury have been used for quantification of methylation rates in pure cultures (Table 3). At 1 ppm of added mercuric chloride we found that methylation in Clear Lake sediments was saturated (42) and elected to apply this saturating level in the present study to facilitate comparison between strains. Comparison of per-cell methylation rates may be complicated by the toxicity of divalent mercury and the resulting suppression of specific growth rates, as we observed both for strain CLFeRB and *D. propionicus* strain 1pr3.

Rates of methylation by sulfate-reducing bacteria have been proposed to be constrained by the rate of entry of membrane-permeable soluble mercury-sulfide complexes (4, 28, 29). By contrast, iron-reducing bacteria, including strain CLFeRB, might be able to initiate methylation via two separate mechanisms. The dissolution of sorbed mercury that could accompany reduction of solid-phase iron has been described above. Alternatively, as demonstrated by the work of Lower et al. (39), one mode of metal reduction is believed to involve direct reduction of the mineral surface by iron-reducing bacteria, and we speculated that this might give direct access to sorbed mercury for methylation independent of the requirement for soluble mercury. It should be noted that at least three strains of sulfate-reducing bacteria have the capacity to interact via respiration with metallic iron (14). To the extent that this finding becomes general, it offers the possibility of more types of microbial control on metal cycling with the potential to impact methylation.

Microbial phylogenetics and mercury methylation. Dissimilatory sulfate reduction, which has been repeatedly described in the literature as the metabolism characterizing dominant environmental methylators, is distributed across the tree of life and includes the low-G+C *Firmicutes* and the δ-subclass of the *Proteobacteria* (55); however, environmentally significant methylation has been demonstrated only in sulfate-reducing bacteria associated with δ-subclass of the *Proteobacteria* (12, 34, 65). The methylating strain CLFeRB1 discussed here is clearly a member of the family *Geobacteraceae* and is an iron reducer based on its nearest neighbors (Fig. 1). Nonetheless, its slightly more distant neighbors among the δ-subclass of the *Proteobacteria* encompass sulfate-reducing bacteria (e.g., orders *Desulfovibrionales* and *Desulfobacterales*) that are known mercury methylators, including *D. propionicus* strain 1pr3, studied here. Several diverse taxa are able to grow via dissimilatory iron reduction, and phylogenetic distinctions between bacteria that utilize various electron acceptors or forms of the same acceptor (e.g., aqueous- versus solid-phase Fe^{3+}) are sometimes blurred (38). Although there are a number of dissimilatory iron-reducing bacteria within the family *Geobacteraceae* that grow on elemental sulfur, none has been reported to be capable of dissimilatory sulfate reduction (38). Two sulfate

reducers have also been found to grow via reduction of iron (62), with *D. propionicus* strain 1pr3 specifically being able to grow with ferric oxyhydroxides as an oxidant (24). Although strain CLFeRB has not yet been tested for the ability to respire with sulfate, its growth in the present study in a sulfate-free medium (with cysteine as the sole source of sulfur) establishes firmly that mercury methylation occurred during growth as an iron reducer rather than as a cryptic sulfate reducer. In the future, when the capacity for active mercury methylation has been more broadly surveyed among the δ-subclass of the *Proteobacteria* and dissimilatory iron reducers, it will be interesting to see whether the capacity to methylate mercury correlates with versatility (or lack thereof) regarding electron acceptor use or with phylogenetic affiliation.

Wider implications. Dissimilatory iron reduction is becoming increasingly recognized as an influential process in certain anomalous iron-rich marine sediments and in iron-rich freshwater sediments in general, where up to 50 to 70% of buried organic carbon can be oxidized via this process (30, 56, 63). The possibility that solid-phase ferric iron is an electron acceptor that is generally important for microbial methylation of mercury might alter the way we view the vertical distribution of mercury methylation in freshwater sediments. If the discussion is restricted to neutral-pH freshwater sediments, i.e., those not receiving advective or diffusive input of acid-rock drainage, sulfate reduction is confined to the upper 5 to 10 cm. The relatively few studies of this process for freshwater sediments reached this conclusion based both on activity measurements and pore water profiles of sulfate (1, 42). By contrast, for neutral-pH Clear Lake sediments bioturbation may impact the upper 10 to 30 cm (60), with the potential to transport iron oxides to those depths, which in turn would expand the zone over which methylmercury is produced. Our preliminary results, which suggest that ferric oxyhydroxides are at least as effective as soluble ferric iron at promoting methylation in pure culture, emphasize the potential importance of this solid-phase electron acceptor in studies of methylation by natural populations of microbes. Additionally, the tendency of divalent mercury to become sorbed into solid-phase iron oxyhydroxides emphasizes that the behavior of iron reducers under various experimental regimens of added mercury may be different from that displayed by sulfate-reducing bacteria, which, as noted above, generally decrease their per-cell methylation rates as added mercury concentrations decrease.

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